

Microbial community composition and enzyme activities in a sandy loam soil after fumigation with methyl bromide or alternative biocides

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Abstract

A sandy loam soil was fumigated in microcosms for 24 h with methyl bromide and chloropicrin (MeBr+CP), propargyl bromide (PrBr), combinations of 1,3-dichloropropene and CP (InLine), iodomethane and CP (Midas), an emulsifiable concentrate of CP (CP-EC), or methyl isothiocyanate (MITC). The effects of these pesticides on fatty acid methyl ester (FAME) profiles and selected enzymatic activities were evaluated in fumigated soils and a nonfumigated control at 1, 3, 7, 14, 21, 28, and 90 days post-fumigation. Bacterial (*a15:0*, *i15:0*, *i16:0*, *cy17:0*, *a17:0* and *i17:0*) and fungal (*18:2ω6*, *18:3ω6*, *18:1ω9*) FAMES were initially (1 day post-fumigation) reduced by fumigation with CP-EC, InLine, and Midas. Microbial communities of soils fumigated with MeBr+CP, MITC, and PrBr resembled those of the control soil. At 14–28 days post-fumigation, FAME profiles were changed in all fumigated soils relative to the control, with the exception of soils treated with MITC. At 90 days post-fumigation, FAME profiles suggested that actinomycetes (10 Me 16:0, 10 Me 17:0, 10 Me 18:0) and Gram-positive bacteria may recover preferentially after fumigation with most of the pesticides studied. Among the fumigants tested, InLine, Midas, and CP-EC had a higher potential to alter the microbial community structure in the longer term than MeBr+CP, PrBr and MITC, with MITC having the least effect. Soil enzyme activities in fumigated microcosms were significantly ($P \leq 0.037$) different from the nonfumigated soil, with the exception of β -glucosidase in soils treated with PrBr and MITC, and dehydrogenase in MeBr+CP-fumigated soils. Over the 90-day study, soil fumigation (average of all fumigants and sampling dates) reduced the activities of arylsulfatase (62%), dehydrogenase (35%), acid phosphatase (22%), and β -glucosidase (6%), suggesting that S mineralization in soils and the total oxidative potential of microorganisms were more affected by fumigation than P and C mineralization. This study also indicates that soil fumigation with MeBr+CP alternative biocides has the potential to alter microbial communities and important key reactions involved in nutrient transformation.

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1. Introduction

Preplant soil fumigation with methyl bromide (CH_3Br , MeBr) and chloropicrin (CCl_3NO_2 , CP) has been used widely around the world to control insects, nematodes, weeds, and pathogens such as *Phytophthora cactorum*, *P. fragariae*, *Verticillium dahliae* and *Colletotrichum acutatum* in many vegetable, fruit, nut, ornamental and nursery crops (Ajwa et al., 2003b; Tanaka et al., 2003). A great portion of the MeBr can potentially escape into the atmosphere during or shortly after the application, contributing to the depletion of the stratospheric ozone layer (Yung et al., 1980; Prather et al., 1984). In

accordance with the Montreal protocol, the import and manufacture of MeBr in the USA and other developed countries will be banned by 2005, after stepwise reductions in 1999, 2001, and 2003 (USEPA, 1993; UNEP, 1997). The loss of MeBr will greatly affect agricultural, silvicultural, and horticultural production unless safe and efficacious alternatives are found. Commercially available alternatives to MeBr are CP, 1,3-dichloropropene ($\text{C}_3\text{H}_4\text{Cl}_2$, 1,3-D), InLine (1,3-D plus CP), and methyl isothiocyanate (MITC) generators such as sodium methyldithiocarbamate ($\text{CH}_3\text{NHCSS Na}$, trade name metam sodium) and potassium methyldithiocarbamate ($\text{CH}_3\text{NHCSS K}$, trade name metam potassium) (Ajwa et al., 2003b). Currently, experimental chemical alternatives to MeBr are iodomethane (CH_3I , trade name Midas) and propargyl bromide ($\text{C}_3\text{H}_3\text{Br}$, PrBr) (Ajwa et al., 2001; 2003b).

Previous studies provide information about the biological degradation of various alternative fumigants in soil (Miller et al., 1997; Gan et al., 1999; 2000; Di Primo et al., 2003) and

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their efficacies against soilborne pathogens and weeds relative to MeBr+CP combinations (Fennimore et al., 2003; Haar et al., 2003). Most of these fumigants are known to have a broad biocidal activity (Anderson, 1993), but their impacts on the structure and functionality of the soil microbial community are largely unknown. Soil microorganisms control nutrient availability and pesticide degradation and thus, influence soil functioning and productivity of agricultural systems.

A popular technique to detect changes within soil microbial groups is the extraction of fatty acids derived from the phospholipid components of the cellular membranes of microorganisms. Fatty acid methyl ester (FAME) profiles of soils can be compared using multivariate statistical techniques to reveal differences in microbial communities (Macalady et al., 1998). Comparisons of the abundance of groups of FAMEs presumed to be unique for bacteria (i.e. *a15:0*, *i15:0*, *a17:0*, and *i17:0*), mycorrhiza (i.e. *18:1 ω 9c*, and *16:1 ω 5c*), and/or fungi (i.e. *18:2 ω 6c*, and *18:3 ω 6c*) can provide information on community composition and relative microbial group abundance. Although the use of marker FAMEs has been criticized because fatty acids may occur across taxa, some fatty acids, such as bacterial markers, are rarely found in other microbial groups (Cavigelli et al., 1995; Bossio et al., 1998; Zelles, 1999). Moreover, marker bacterial or fungal FAMEs have been shown to correlate well with soil bacterial or fungal biomass measurements (Ndiaye et al., 2000; Schutter et al., 2001a; Acosta-Martínez et al., 2004b). The extraction and identification of fatty acid methyl esters by using the commercially available MIDI protocol and gas chromatograph-software system (Microbial ID, Inc. [MIDI], Newark, Delaware, USA) provides a fast, simple, cost effective, and reproducible method (Cavigelli et al., 1995; Ibekwe and Kennedy, 1999; Schutter and Dick, 2001; Acosta-Martínez et al., 2004a,b).

The FAME profiles of soil microbial communities were shown to be sensitive to management (Zelles et al., 1992; Acosta-Martínez et al., 2004a,b), seasonal changes (Scholz and Boon, 1993), rhizosphere effects (Tunlid et al., 1985; 1989), pollution (Bååth et al., 1992), addition of composts (Tunlid et al., 1989) and pesticides (Macalady et al., 1998; Ibekwe et al., 2001). By using phospholipid fatty acids (PLFA) and denaturing gradient gel electrophoresis (DGGE) analysis, changes in heterotrophic activity and fatty acid composition of microbial communities were found after soil fumigation with MeBr, MITC, 1,3-D, and CP (Ibekwe et al., 2001) and metam sodium (Macalady et al., 1998), indicating that fumigants have the potential to alter nutrient cycling in soils.

Kandeler et al. (1996) suggested that the composition of the microbial community strongly affects the potential of a soil for enzyme-mediated substrate catalysis. Consequently, changes in microbial diversity in fumigated soils may also reduce microbial functionality. There is growing evidence that most organisms are functionally redundant and that the functional characteristics of component species are at least as important as the number per se for maintaining essential processes (Andren and Balandreau, 1999; Bardgett and Shine, 1999). Because microbial functional diversity includes many different metabolic processes, enzyme activities that control key metabolic

pathways in soil can be measured and used as an index for microbial functional diversity (Nannipieri et al., 2002). Understanding the dynamics of key enzymatic processes in soil relative to environmental changes such as pesticide applications may allow better prediction of organic matter and nutrient turnover in intensively managed agricultural soils. Among the key enzymatic reactions in soils are those catalyzed by acid phosphatase (EC 3.1.3.2), β -glucosidase (EC 3.2.1.21), and arylsulfatase (EC 3.1.6.1) reflecting the potential of a soil to mineralize organic P, C and S compounds to plant available forms, respectively. In addition, the activities of dehydrogenases (EC 1.1.) are known as a measure for the total oxidative activities of soil microorganisms. Soil enzyme activities provide insights into the nutritional status of soils associated with management such as cropping systems (Kandeler et al., 1999; Klose et al., 1999; Klose and Tabatabai, 2000; Acosta-Martínez et al., 2003; 2004a,b), pollution (Nannipieri, 1994; Deng and Tabatabai, 1995; Klose et al., 2003; 2004) and pesticide application (Klose and Ajwa, 2004).

The evaluation of fumigant effects on the structure and functionality of the soil microbial community is crucial to gain a more holistic understanding of the biocidal activity of these pesticides and their potential environmental impact. Therefore, our study investigated the impacts of the most promising biocides in replacing the standard MeBr+CP application (Ajwa et al., 2003b) on soil microbial functional and structural diversity at 1, 3, 7, 14, 21, 28, and 90 days post-fumigation under laboratory conditions. Our objectives were to determine the biocidal effects of the five alternative fumigants InLine, CP-EC, PrBr, Midas, and MITC relative to MeBr+CP and a control soil by (1) monitoring changes in the microbial community structure as indicated by FAME profiles and selected bacterial and fungal marker fatty acids, and in several enzymatic key reactions involved in soil nutrient transformation, and (2) evaluating whether changes in the composition of the microbial community will directly affect the microbial functionality as indicated by enzymatic activities over the three month-study.

2. Materials and methods

2.1. Fumigant concentrations

Fumigant rates were based on recommended field application rates ranging between 202 and 926 kg ha⁻¹ y⁻¹ for MeBr+CP and alternative biocides. Assuming that the fumigant solutions distribute evenly over the entire soil volume (15 cm soil depth, 1.7 g cm⁻³ bulk density), these application rates are equivalent to 80–363 mg fumigant kg⁻¹ soil (dry wt). Fumigants used, application rates, properties and concentrations of the fumigants are summarized in Table 1.

2.2. Soil type and microcosm study

Soil samples were collected from the top 15 cm on the University of California Davis Agricultural Experiment Station in the central coastal region in Watsonville (121°50'W,

Table 1

Fumigant properties, vendor, and application rates used in the microcosm study on a sandy loam soil

Soil fumigant ^a	Vendor	Percent a.c. ^b	Density g cm ⁻³ (20 °C)	Field application rate kg ha ⁻¹ y ⁻¹	Concentration mg kg ⁻¹ soil (dry wt)
Control	NA ^c	NA	NA	0	0
MeBr+CP	Tri-Cal Inc., Hollister, Calif., USA	67+33	1.73 ^d	420	165
PrBr	Albemarle Corporation Baton Rouge, Louisiana, USA	80	1.08	202	79
InLine	DowAgroSciences, Redek, North Carolina, USA	61+33	1.21	448	176
Midas	Arvesta Corporation, San Francisco, Calif., USA	50+50	1.98	448	176
CP-EC	Niklor Chemical Co., Long Beach, Calif., USA	96	1.65	336	132
MITC	Aldrich, Milwaukee, Wisconsin, USA	97	1.07	450	177

^a Abbreviations: MeBr, methyl bromide; CP, chloropicrin; EC, emulsifiable concentrate; InLine: mixture of 1,3-D, 1,3-dichloropropene and CP; Midas, mixture of iodomethane and CP; MITC, methylisothiocyanate; PrBr, propargyl bromide; control, nonfumigated soil.

^b a.c., Active component.

^c Not applicable.

^d Density of MeBr at 0 °C.

36°54'N), California, USA, on a plot with no known history of fumigant treatment. The soil is classified as an Elder sandy loam (coarse-loamy, mixed, thermic, Cumulic Haploxeroll), with a mean particle size distribution of 62% sand, 26% silt, and 12% clay. The pH was 7.75 (H₂O) and 7.08 (0.01 M CaCl₂), and the organic C content was 6 g kg⁻¹ soil. Field moist soil was passed through a 2-mm sieve. The moisture content of the soil was adjusted to 15% to ensure optimal fumigant dissipation. Soils were mixed and preconditioned at 25 °C for 48 h before being used in the study.

Soils were placed in microcosms consisting of glass jars containing 500 g soil (dry wt). Jars were sealed with an air-tight lid equipped with rubber septa. The experimental design consisted of six fumigants and a control in three replicated microcosms. The fumigants were added by a syringe as freshly prepared aqueous solutions sufficient to bring soil moisture contents to field capacity. Microcosms were exposed to the fumigants for 24 h. Exposure time was selected based on field studies indicating that the maximum concentration of MITC, CP, and 1,3-D in soil is reached within 18–24 h (Ajwa and Trout, 2000; 2004; Ajwa et al., 2003a). Afterwards the jars were uncapped and vented under the hood for 5 min, soils were mixed and jars vented again for another 30 min until any remaining volatile fumigant was released from the microcosms. Preliminary studies showed that >95% of the fumigants in microcosms dissipates within 30 min after opening the jars.

Soil samples were collected at 1, 3, 7, 14, 21, 28, and 90 days post-fumigant application and stored at 4 °C for microbial community analysis and enzyme assays. Soil microbial analysis was completed within one week of sampling. Soil moisture contents in the microcosms were maintained gravimetrically.

2.3. FAME profiles

Fatty acid methyl esters were extracted from the microcosm soil samples using the procedure described for pure culture isolates by the Microbial Identification System [Microbial ID (MIDI), Inc., Newark, DE, USA] as previously applied to soil analyses (Cavigelli et al., 1995; Ibekwe and Kennedy, 1999;

Acosta-Martínez et al., 2004a,b). Three-gram soil samples were treated according to the four steps of the MIDI protocol for biological samples: (1) saponification of fatty acids at 100 °C with 3 ml 3.75 M NaOH in aqueous methanol (methanol: water ratio=1:1) for 30 min, (2) methylation (esterification) at 80 °C in 6 ml of 6 M HCl in aqueous methanol (1:0.85) for 10 min, (3) extraction of the FAMES with 3 ml of 1:1 (vol/vol) methyl-tert-butyl ether/hexane by rotating the samples end-over-end for 10 min, and (4) washing of the solvent extract with 1.2% (wt/vol) NaOH by rotating the tubes end-over-end for 5 min. The FAMES were analyzed in a 6890 GC Series II (Hewlett Packard, Wilmington, DE, USA) equipped with a flame ionization detector and a fused silica capillary column (25 m×0.2 mm) using ultra high purity hydrogen as the carrier gas. The temperature program was ramped from 170 to 250 °C at 5 °C min⁻¹. The FAMES were identified, and their relative peak areas (percentage) were determined with respect to the other FAMES in a sample using the MIS Aerobe method of the MIDI system. The FAMES are described by the number of C atoms, followed by a colon, the number of double bonds and then by the position of the first double bond from the methyl (ω) end of molecules, and cis and trans isomers are indicated by c or t, respectively. Branched fatty acids are indicated by the prefixes *i* and *a* for iso and anteiso, respectively.

2.4. Enzyme assays

Potential dehydrogenase activity was assayed by incubating 5 g moist soil amended with glucose (16 mg g⁻¹ dry soil, finely ground and mixed with talcum powder (1:3 wt/wt)) with 5 ml of triphenyltetrazolium chloride (TTC) solution (0.8%, dissolved in Tris buffer (0.1 M, pH 7.6)) at 30 °C for 24 h. Controls contained only 5 ml Tris buffer (0.1 M, pH 7.6). Triphenyl formazan (TPF) produced was extracted with methanol and estimated colorimetrically (Thalman, 1968). Results are expressed as mg of TPF released kg⁻¹ soil 24 h⁻¹.

The activities of acid phosphatase, β-glucosidase and arylsulfatase were assayed on 1-g oven-dry equivalents of buffered soil solutions incubated for 1 h at 37 °C after addition of the enzyme-specific substrate solution (Tabatabai, 1994).

The product of all reactions, *p*-nitrophenol (PN), was measured colorimetrically (Tabatabai, 1994) and is expressed as mg of PN kg⁻¹ soil h⁻¹.

2.5. Data analysis

Two-way analysis of variance (ANOVA) was performed to compare groups of sampling points defined a priori by the fumigant treatment, sampling time, and the interaction between both on total FAME abundance, and on bacterial and fungal FAME groups. Indicator FAMES for soil fungi (18:1ω9c, 18:2ω6c and 18:3ω6c), Gram-positive (Gm+) bacteria (i14:0, i15:0, a15:0, i16:0, i17:0, a17:0), Gram-negative (Gm-) bacteria (cy17:0), and actinomycetes (10 Me16:0 and 10 Me17:0) were evaluated with the PC-ORD statistical software (version 4) in order to compare the effects of alternative methyl bromide fumigants on the community structure compared to the control after fumigation (McCune and Mefford, 1999). The data were examined using nonmetric multidimensional scaling (NMS) with the Sorensen distance measure (Kruskal, 1964; Mather, 1976). The NMS analyses were performed using the 'slow and thorough' setting in the autopilot mode of the PC-ORD statistical software. Random starting configurations were seeded by the computer's clock to obtain forty runs with real data. Monte Carlo simulations were conducted using 50 randomized runs and a stability criterion of 0.0001. The number of dimensions chosen in the model was assessed by comparing the NMS runs with the real data to Monte Carlo simulations. The proportion of variation represented by each axis was assessed by calculating the coefficient of determination (r^2) between distances in the ordination space and Sorensen distances in the original distance matrix.

Percentage of changes in the abundance of enzyme activities due to fumigant treatment was calculated as $[(A - B)/B] \times 100$, where *A* is the value of the fumigated soil and *B* is the value of the control soil. Enzyme activities were assayed for one control and in duplicate otherwise. All data were calculated on an oven-dry (105 °C) basis and are given as arithmetic means of three replicates for each microcosm. Two-way analysis of variance (ANOVA) was used to assess the effects of the fumigant treatment and sampling time, and differences among means were calculated with Fisher's least significant differences (LSD) test. Canonical discriminant analysis (DA) was performed for enzyme data for the separation of distinct groups within the multidimensional data swarm. The independent variables (i.e. enzyme activities) are used as predictors of group membership. For the DA, each discriminant function (DF) discriminates among groups by extracting a portion of the variance in the original data, with the greatest amount of variance for the first DF, and as much of the remaining variability as possible for each succeeding DF. DA was performed separately for the effects of treatment and days after fumigation. The results of the DA were used to reveal which of the environmental parameters predicted the first two PCs for enzyme activity data. ANOVA, LSD test and DA were performed with SPSS (version 10.07 for Windows).

3. Results

FAME profiles and enzyme activities were determined in soil samples taken prior to fumigation. Results of this sampling date were similar to the untreated control at 1 day post-fumigation (data not shown).

Table 2

Probability levels of the two-way ANOVA for the effects of fumigants and days post-fumigation on soil FAMES using fumigant treatment and sampling time (d post-fumigation) as main factors

FAMES	Overall	Difference in abundance of FAMES in control vs. tested fumigants ^a					
		MeBr+CP	PrBr	InLine	Midas	CP-EC	MITC
Total							
Fumigant	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
Days post-fumigation	$P < 0.001$						
Fumigant*Days post-fumigation	$P < 0.001$						
Bacterial indicators ^b							
Fumigant	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.01$
Days post-fumigation	$P < 0.001$						
Fumigant*Days post-fumigation	$P < 0.001$						
Fungal indicators ^c							
Fumigant	$P < 0.001$	n.s.	n.s.	$P < 0.06$	$P < 0.001$	$P < 0.1$	n.s.
Days post-fumigation	$P < 0.01$						
Fumigant*Days post-fumigation	$P < 0.1$						
Mycorrhizal indicator ^d							
Fumigant	$P < 0.001$	n.s.	$P < 0.018$	$P < 0.002$	$P < 0.001$	$P < 0.001$	n.s.
Days post-fumigation	n.s.						
Fumigant*Days post-fumigation	n.s.						

^a According to the students LSD test (n.s., not significant).

^b i15:0, a15:0, a17:0, i17:0, cy17:0, i16:0.

^c 18:3ω6c, 18:1ω9c, 18:2ω6c, 16:1ω5c.

^d 16:1ω5c.

3.1. FAME profiles

One hundred and forty-five fatty acids were identified by the FAME method-MIDI software, and one hundred and thirteen fatty acids were commonly found in both the fumigated and nonfumigated soil samples. The most abundant fatty acid was 16:0 (avg.: 19.60%, S_D : 8.16%) followed by 18:1 ω 9c (avg.: 7.27, S_D : 2.74), 18:2 ω 6c (avg.: 4.13%, S_D : 4.49%), *a*15:0 (avg.: 3.30%, S_D : 0.92%), and *i*15:0 (avg.: 4.15%; S_D : 1.26%), which accounted for approximately 38% of the total peak area in the FAME profiles of the soils studied (data not shown).

Multivariate analyses of variance revealed that total FAME abundance was affected by soil fumigation, sampling time

(days post-fumigation), and the interaction between both factors ($P < 0.001$) (Table 2). The relative abundance of indicator bacterial (*i*15:0, *a*15:0, *i*16:0, *a*17:0, *i*17:0, *cy*17:0) FAMES was significantly ($P < 0.001$) affected by the fumigant, days post-fumigation, and the interaction between both factors. These findings suggest that all fumigants had the potential to change bacterial populations in soil, but the time until the bacteria recovered from the initial effects varied with the fumigant. Fungal FAME indicators (16:1 ω 5c, 18:2 ω 6c, 18:1 ω 9c, and 18:3 ω 6c) were significantly affected by soil fumigation and days post-fumigation ($P \leq 0.01$). Fungal FAMES in soils fumigated with Midas deviated significantly ($P < 0.001$) from nonfumigated soils. Fumigated soils, with the

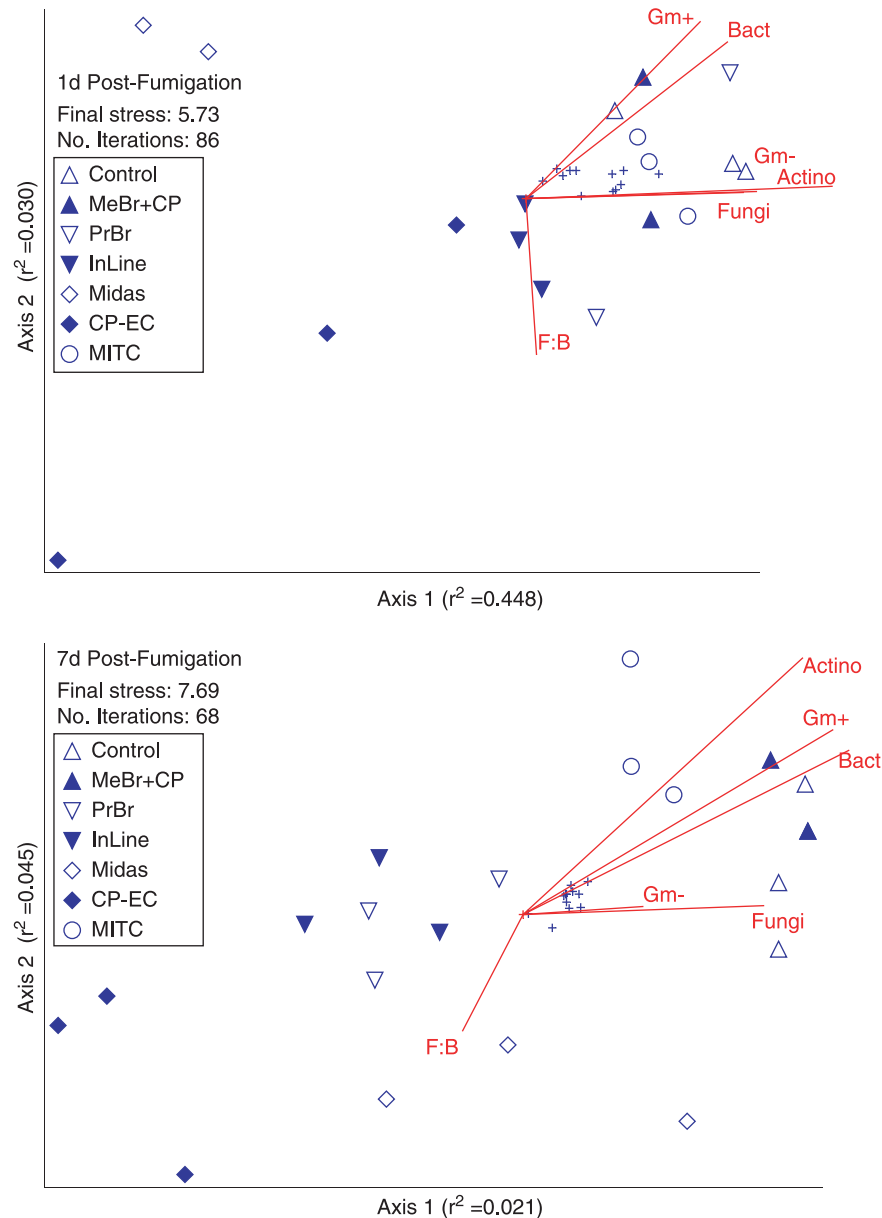


Fig. 1. Results from NMS ordination using the Sorensen distance measure of the effects of different fumigants on microbial community structure at 1 and 7 days post-fumigation. Each point in the ordination is a different fumigant treatment. The second matrix variables are overlaid on the NMS as a joint plot. The angle and length of the line indicates the direction and strength of relationship. Abbreviations: MeBr, methyl bromide; PrBr, propargyl bromide; InLine, 1,3-dichloropropene plus chloropicrin; Midas, Iodomethane plus chloropicrin; CP-EC, chloropicrin, emulsifiable concentrate; MITC, methyl isothiocyanate; Actino, actinomycetes; Gm +, Gram-positive bacteria; Gm –, Gram-negative bacteria; F:B, fungi:bacteria ratio.

exception of the MeBr+CP and the MITC treatment, consistently had lower relative abundances of the fungal FAME 16:1 ω 5c.

In order to explain differences in specific groups of the soil microbial community for each sampling time, FAME indicators of Gm+ and Gm– bacteria, fungi, and actinomycetes were presented in NMS ordination plots

(Figs. 1 and 2). At 1 day post-fumigation, fungi and actinomycetes were positively correlated with axis 1 ($r^2=0.448$), and the fungi:bacteria (F:B) ratio was negatively correlated with axis 2 ($r^2=0.030$) (Fig. 1). Lower relative abundances of Gm+ and Gm– bacteria and a higher F:B ratio were found in soils fumigated with CP-EC, InLine and PrBr, while Midas-fumigated soils revealed lower abundances

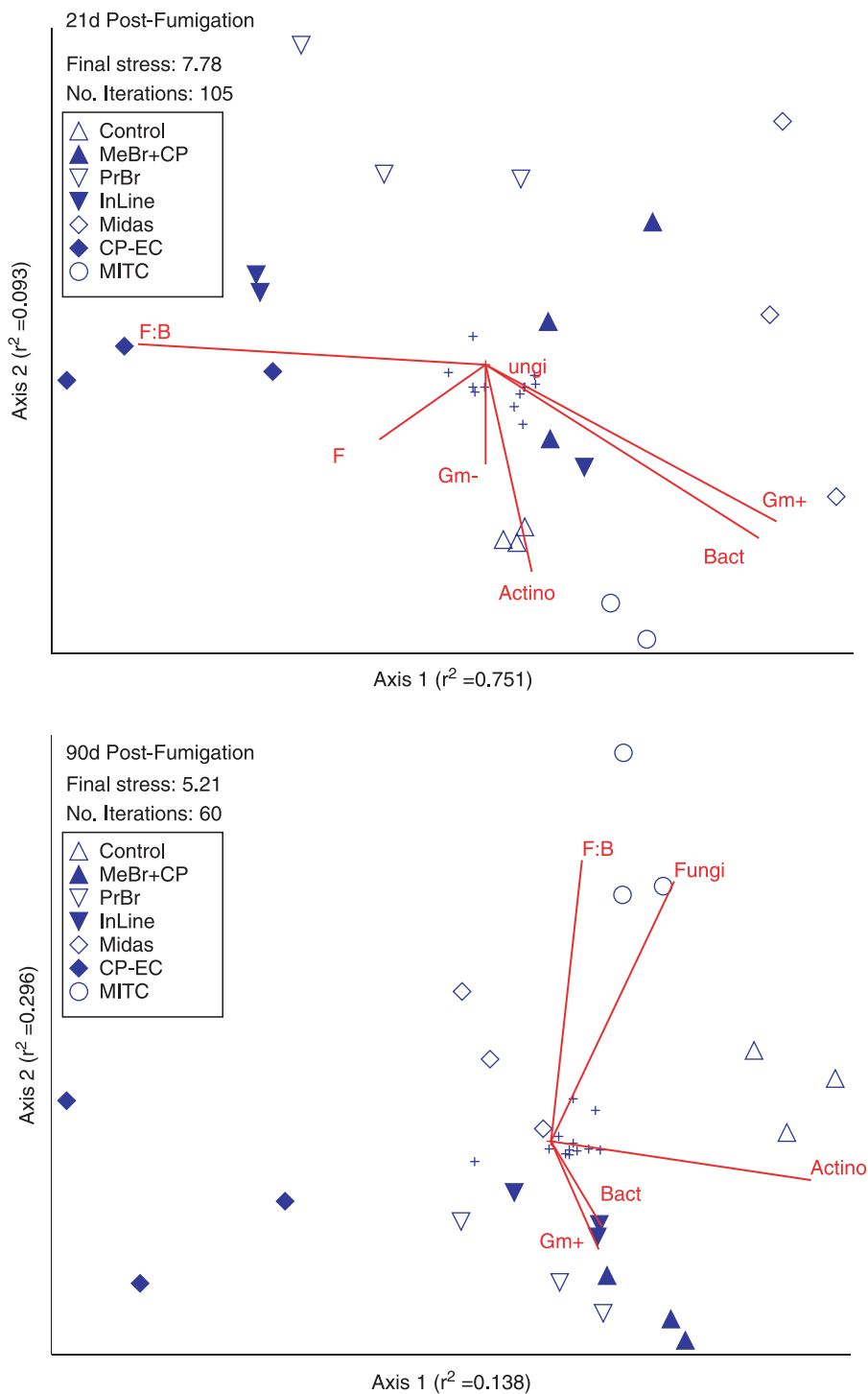


Fig. 2. Results from NMS ordination using the Sorensen distance measure of the effects of different fumigants on microbial community structure at 21 and 90 days post-fumigation. Each point in the ordination is a different fumigant treatment. The second matrix variables are overlaid on the NMS as a joint plot. The angle and length of the line indicates the direction and strength of relationship. Abbreviations: see Fig. 1.

of actinomycetes and fungi when compared to the control and soils fumigated with MeBr+CP and MITC. Generally, soils treated with CP-EC and Midas showed the greatest shifts in the microbial community structure compared to the control at 1 and 3 days (data not shown) post-fumigation. At 7 days post-fumigation, soils fumigated with CP-EC, InLine, PrBr and Midas showed lower relative abundances of bacteria (especially Gm+), actinomycetes, and higher F:B ratios compared to the control (Fig. 1). Microbial groups in soils fumigated with MeBr+CP and MITC were similar to those of the nonfumigated control. Midas-fumigated soils showed higher relative abundances of fungi, and a higher F:B ratio than the control soil. At 21 days post-fumigation, soils fumigated with MeBr+CP and Midas showed a lower relative abundance of fungi and F:B ratios (Fig. 2). Soils fumigated with PrBr, InLine, and CP-EC had lower relative abundances of bacteria and actinomycetes than the control. Similar results were observed at 14 and 28 days post-fumigation (data not shown). At 90 days post-fumigation, abundance of bacteria (Gm+ and Gm-) was negatively correlated along axis 2 ($r^2=0.296$), indicating lower bacterial populations in soils fumigated with PrBr and InLine (Fig. 2). Fungal groups were positively correlated along axis 2, suggesting a lower fungal abundance in soils treated with CP-EC, PrBr and Midas compared to the control. Soils fumigated with MeBr+CP and PrBr expressed a lower F:B ratio than control soils and soils treated with MITC.

3.2. Enzyme activities

The activities of acid phosphatase, arylsulfatase, β -glucosidase and dehydrogenase were significantly ($P<0.001$) affected by the fumigant treatment, the sampling time (days post-fumigation), and the interactions between these two main factors. The activities of the studied enzymes in soils from microcosms fumigated with MeBr+CP and the five tested

alternative biocides were significantly ($P\leq 0.037$) different from the nonfumigated soil, with the exception of β -glucosidase in microcosms treated with PrBr and MITC, and dehydrogenase in MeBr+CP-fumigated soils.

Canonical discriminant analysis on enzyme data separated four groups of treatment effects, which are (1) the control group (66.7% of predicted group membership), (2) the group containing microcosms fumigated with MeBr+CP, PrBr, Midas and InLine, (3) the CP-EC group (66.7% of predicted group membership), and (4) the MITC group (81.0% of predicted group membership) (Table 3). These four groups were significantly separated along DF1 (Wilks' $\lambda=0.330$, $P<0.001$) and DF2 (Wilks' $\lambda=0.715$, $P<0.001$), with both functions explaining 96.5% of the variation among the enzyme activity data (Fig. 3(A)). Discriminant analysis further separated four groups of sampling-time-effects (days post-fumigation). Enzyme activities were similar in microcosms at 1, 3, 7, and 21 days post-fumigation (group 1), and distinct from those at 14 days (group 2), 28 days (group 3), and 90 days (group 4) post-fumigation (Table 3). These groups were significantly separated along DF1–DF3 (Wilks' $\lambda\leq 0.647$, $P<0.001$) (Fig. 3(B)). The three discriminant functions explained 99.8% of the variation among the enzyme data. These results suggest that changes in enzyme activities imposed by soil fumigation continued to the end of the 90-day incubation period, and that differences were most pronounced at 14 days post-fumigation.

Soil fumigation with MeBr+CP and alternative biocides decreased acid phosphatase activity between 5 and 80% up to 14 days post-fumigation (Table 4). At 21–90 days post-fumigation, phosphatase activity in fumigated soils slightly increased but remained lower than the control with the exception of the microcosms treated with MeBr+CP at 21 days, and with PrBr and InLine at 90 days post-fumigation. Fumigation with MITC had the greatest impact on acid phosphatase. The effects of the other biocides tested on this enzyme activity were similar.

Table 3
Classification results of the canonical discriminant analysis for the effects of soil treatment and sampling time (d post-fumigation)

Main factors	Predicted Group Membership (%)						
Treatment ^a	Control	MeBr+CP	PrBr	InLine	Midas	CP-EC	MITC
Control	66.7	28.6	0	0	0	0	4.8
MeBr+CP	14.3	38.1	9.5	4.8	14.3	14.3	4.8
PrBr	0	19.0	14.3	38.1	4.8	14.3	9.5
InLine	0	9.5	4.8	33.3	38.1	9.5	4.8
Midas	0	14.3	4.8	28.6	19.0	19.0	14.3
CP-EC	0	4.8	4.8	0	9.5	66.7	14.3
MITC	0	4.8	14.3	0	0	0	81.0
Date ^b	1d	3d	7d	14d	21d	28d	90d
1d	28.6	57.1	0	0	14.3	0	0
3d	28.6	57.1	0	0	14.3	0	0
7d	9.5	28.6	28.6	0	14.3	19.0	0
14d	0	0	0	95.2	0	4.8	0
21d	0	0	28.6	0	47.6	14.3	9.5
28d	0	0	0	0	0	100	0
90d	0	0	0	0	4.8	10	85.7

^a 45.6% of original grouped cases correctly classified.

^b 63.3% of original grouped cases correctly classified.

Arylsulfatase activity was generally reduced (up to 99%) in fumigated microcosms relative to the control over the whole 90 day study. During the first 14 days of incubation the reduction of arylsulfatase activity in soils fumigated with PrBr, InLine, Midas, and MITC was more pronounced than in soils fumigated with MeBr+CP and CP-EC. At 90 days post-fumigation, arylsulfatase activity in microcosms fumigated with PrBr was 74% higher than those in the control soil, and recovered in microcosms treated with Midas almost to the values of the control soil. Arylsulfatase activity remained significant lower until the end of the 90-day study in soils

fumigated with MeBr+CP, InLine and CP-EC (only 86, 28, and 71% of the control soil).

The β -glucosidase activity was between 13 and 51% lower in all fumigated soils compared to the control between 1 and 7 day post-fumigation. At 14 days post-fumigation, β -glucosidase activity increased by 45–90% in microcosms fumigated with the alternative biocides relative to the control. At 21–90 days post-fumigation, the responses of β -glucosidase activity to the tested biocides varied from an increase (up to 47%, InLine, 28 days post-fumigation) to a decrease (up to 30%, PrBr, 28 days post-fumigation) (Table 4).

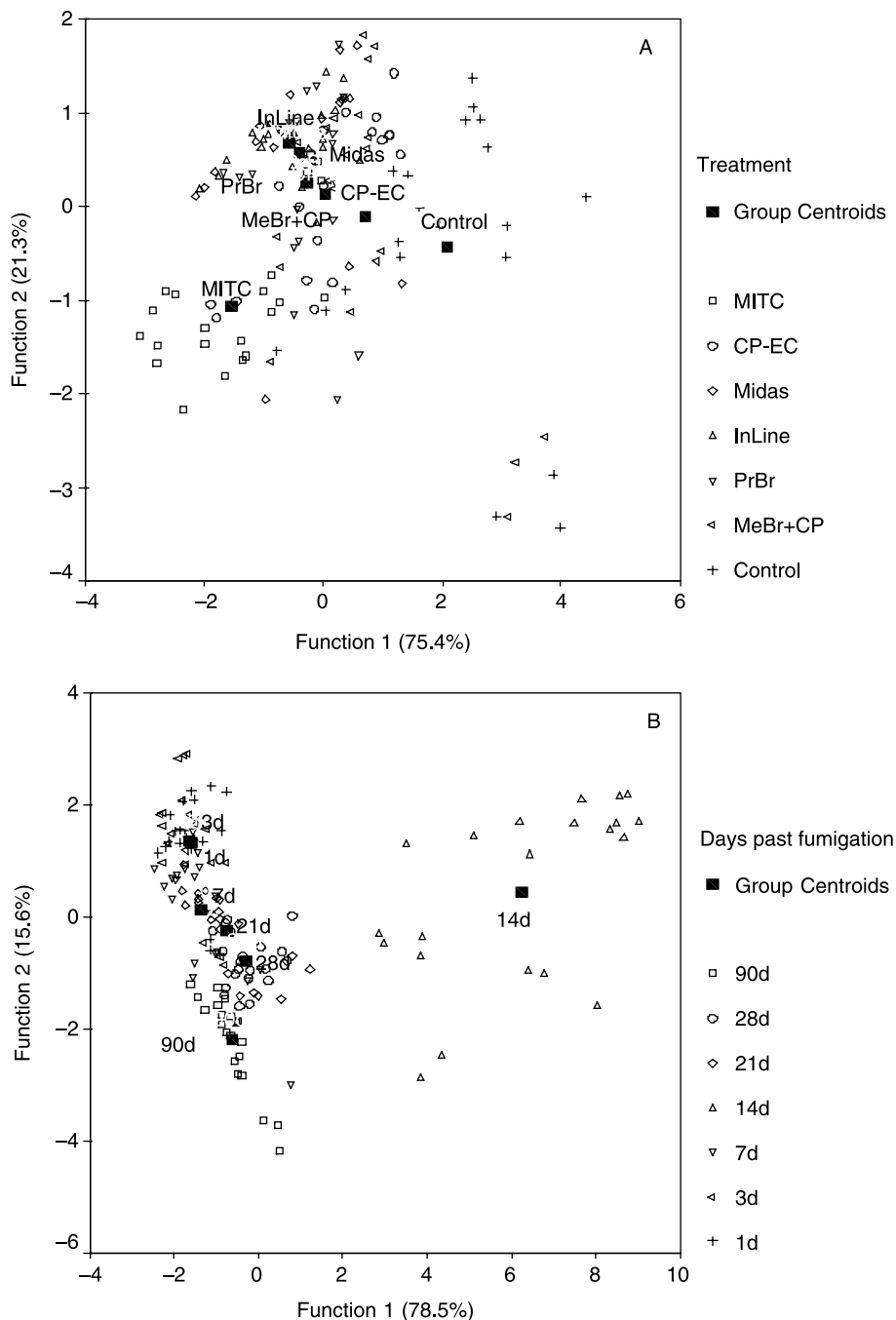


Fig. 3. Canonical discriminant analysis on enzyme data for the separation of the effects of treatment (A) and days post-fumigation (B). These groups were significantly separated along discriminant functions 1 and 2 ($P < 0.001$).

Table 4

Percentage of change in soil enzyme activities in microcosms fumigated with methyl bromide and alternative biocides compared to the non-fumigated soil at 1–90 days post-fumigation

Enzyme activity	Fumigants ^a					
Days post-fumigation	MeBr + CP	PrBr	InLine	Midas	CP-EC	MITC
Acid phosphatase						
1	–20a	–17a	–14a	–9a	–5a	–76b
3	–13a	–25ab	–35b	–20ab	–20ab	–80c
7	–14ab	–18ab	–21b	–17ab	–24b	–70c
14	–30a	–22a	–20a	–20a	–18a	–58b
21	+2a	–30cd	–24abcd	–27bcd	–21abc	–51d
28	–14ab	–20a	–16ab	–7ab	–2b	–19a
90	–22a	+1ab	+58b	–3ab	–27a	–35a
Arylsulfatase						
1	–52ab	–66a	–69a	–69a	–24b	–72a
3	–71a	–94a	–91a	–82a	–47b	–85a
7	–83ab	–87a	–90a	–90a	–34c	–61bc
14	–69ab	–74ab	–84a	–78ab	–57b	–77ab
21	–9a	–69d	–99d	–99d	–79b	–86c
28	+7cd	–64d	–96a	–58ab	–85bc	–95bc
90	–14a	+74b	–72a	–2ab	–29a	–11ab
β-Glucosidase						
1	–28ac	–46bd	–37abd	–32abc	–19c	–51d
3	–34ab	–41a	–26ab	–16b	–13b	–47a
7	–13a	–30a	–15a	–18a	–33a	–51a
14	–11a	+51bc	+80c	+90c	+80c	+45abc
21	–17a	+46b	–19a	–21a	–10a	–11a
28	+1a	–30a	+47a	+39a	+18a	+30a
90	–11ab	–27a	–22ab	–19ab	–5b	–25ab
Dehydrogenase						
1	–49a	–57abc	–50ab	–60b	–67bcd	–79d
3	–37ab	–30a	–9b	–9b	–33d	–30a
7	–69a	–94ab	–98ab	–98b	–70ab	–65ab
14	–21a	–51c	–71c	–69bc	–50bc	–2ab
21	–8a	–15a	–93c	–99c	–51b	–27ab
28	–28a	+5a	–12a	–61a	+103a	+105a
90	+37b	–6ab	0ab	+36b	–45a	–42ab

^a The percentages of change (+, increases; –, decreases) in activities due to fumigation were calculated from $[(A - B)/B] \times 100$, where *A* is the activity value of the fumigated soil and *B* is the activity value of the control (nonfumigated) soil. Means followed by the same letter are not statistically different at $P \leq 0.05$ for the same sampling date using the LSD test.

Dehydrogenase activity was reduced in fumigated soils relative to the control between 1 and 21 days post-fumigation. This enzyme activity was lowest at 7 days post-fumigation with reductions ranging between 65 and 98%. Dehydrogenase activity was up to 105% higher in microcosms fumigated with PrBr, CP-EC and MITC compared to the control soils at 28 days post-fumigation, and slightly recovered in the Midas and InLine treated soils, but not to the control level. After 90 days, the responses of dehydrogenase activities to the different fumigants varied. The enzyme activities in InLine-treated soils were similar to the control, higher in MeBr + CP and Midas-treated soils, and lower in soils fumigated with PrBr, CP-EC and MITC (Table 4).

4. Discussion

Our study showed that among the 113 FAMES found in both fumigated and nonfumigated soils, only two bacterial (*a15:0*, *i15:0*) and fungal (*18:1ω9c*, *18:2ω6c*) FAME indicators and one ubiquitous FAME (*16:0*) accounted for a significant

percentage (38%) of the total peak area in the FAME profiles of the soils. This finding is in agreement with results from a silt-loam fumigated with metam sodium (sodium methyl dithiocarbamate, an MITC generator) in California (Macalady et al., 1998). Among the fumigants studied, InLine, Midas, and CP-EC resulted in stronger shifts in the FAME profiles compared to the control than did PrBr, MeBr + CP, and MITC. Fumigated soils, with the exception of the MeBr + CP and the MITC treatment, consistently had lower relative abundances of the fungal FAME *16:1ω5c*. This observation may be of ecological significance because *16:1ω5c* has been commonly suggested as a marker for arbuscular mycorrhizal fungi (Olsson, 1999; Madan et al., 2002). Our results are consistent with the high sensitivity of mycorrhizal fungi to soil fumigation with pesticides (Iloba, 1978; Trappe et al., 1984; Davis et al., 1996). The reduction of mycorrhizal indicator fatty acids by soil fumigation may be an important factor for management decisions as the numerous benefits of mycorrhizal associations for soil functioning and crop production have been widely recognized (Barea, 1991; Allen, 1992; Acosta-Martínez

et al., 2004a). In agreement with our findings, shifts in soil PLFAs were observed in a microcosm study with a sandy loam one week post-fumigation with MeBr, MITC, 1,3-D, and CP (Ibekwe et al., 2001). The same study found that the PLFA profiles resembled each other and that of the control sample between 8 and 12 weeks post-fumigation. Another microcosm study with metam sodium found shifts in soil FAME profiles compared to the control up to 5 weeks post-fumigation, and no separation among treatments at 18 weeks post-fumigation (Macalady et al., 1998).

Our results indicate that among the alternative fumigants studied, MITC had the least impact on the microbial community structure as its FAME profiles generally resembled those of the control. Fumigation with Midas generally reduced fungal diversity compared to the control, whereas fumigation with InLine, PrBr, and CP-EC reduced bacterial diversity. Ibekwe et al. (2001) reported that the fumigant 1,3-D, a component of InLine, had the least impact on soil bacteria compared to MeBr and other alternative fumigants. Thus, the significant shifts in bacterial FAMES in microcosms treated with InLine in our study demonstrate that the combination of 1,3-D with CP can broaden its biocidal activity.

Low sensitivity of the microbial biomass towards repeated soil fumigation with MeBr + CP, PrBr, InLine, Midas, and CP-EC was observed by Klose and Ajwa (2004). They suggested that the low response of total microbial biomass to soil fumigation may be related to a selective effect on sensitive microbial populations and the growth of resistant species. The latter may feed on cell debris, leading to restructuring of soil microbial populations. However, we found that the effect of different fumigants on microbial community structure and selected microbial groups varied with sampling date (i.e. days post-fumigation), both between and within trophic groups. The low sensitivity of the microbial community structure to fumigation with MITC may suggest that this fumigant targets a specific group of microorganisms that may not be represented by the indicator FAMES for fungi, bacteria and actinomycetes evaluated in this study. Our ANOVA results showing significant shifts in the FAME profiles in MITC-fumigated soils support this hypothesis.

The interpretation of the observed changes in microbial community structure is limited by the fact that fatty acid extraction efficiencies may be poor using the FAME-MIDI method (Macalady et al., 1998) or that fatty acids from clay-organic matter complexes may have also been extracted (Acosta-Martínez et al., 2004a). Compared to PFLA methods, FAME methods have extracted less bacteria indicators and higher fungi indicators (18:2 ω 6c) (Drenovsky et al., 2004). In addition, the trends observed with FAME analyses may not necessarily represent the actual impact of these fumigants on specific microbial species with plant pathogenic potential.

These changes in the microbial community structure were reflected in soil enzyme mediated processes throughout the 90-day study. Only the effects of MITC on microbial community structure diverged from those on soil enzymes. Our results indicate that MITC is more toxic to specific enzyme reactions involved in nutrient cycling carried out by a smaller group of

microorganisms or accumulated enzymes than to the overall microbial community structure as measured by FAME analysis. Discriminant analysis on enzyme data indicated that soil fumigation with the alternative biocides changed important reactions crucial for nutrient transformation, and that enzyme activities in microcosms fumigated with CP-EC and MITC diverged from those treated with the other biocides and the control soil.

Over the 90-day study period, the effects of the tested fumigants on enzyme-mediated processes decreased in the following order: MITC > InLine > Midas > PrBr > MeBr + CP > CP-EC. Our study showed that the activities of dehydrogenase and arylsulfatase were more affected by fumigation than acid phosphatase and β -glucosidase activities. Increases in β -glucosidase activity ranging from 45 to 90% at 14 days post-fumigation could be related to increases in enzyme synthesis and/or a release of this enzyme by less sensitive microbial groups to metabolize cell debris after fumigation. The variations in the responses of different enzyme activities to soil fumigation may be related to the chemical nature of the fumigant (such as pesticide half-life in soil), sources of soil enzymes, and the location of the enzyme in the soil microsite. Ladd and Butler (1975) hypothesized that some enzymes are stabilized in the soil environment by complexes of organic and mineral colloids, and thus, partially protected from denaturing by fumigation.

Amato and Ladd (1988) showed the direct influence of chloroform fumigation on enzyme activities in soils, reporting that dehydrogenase activities were completely inhibited by chloroform fumigation. Chloroform fumigation of soils increased arylsulfatase activity by 57% and decreased the activities of acid phosphatase and β -glucosidase on average by 6 and 22%, respectively (Klose and Tabatabai, 1999, 2002a,b). Schutter et al. (2001b) reported that the activities of dehydrogenase and arylsulfatase were inhibited in field plots fumigated with MeBr + CP and the alternatives PrBr, InLine, Midas and CP-EC one week after soil fumigation, supporting the results of this microcosm study. After 30 weeks, acid phosphatase and arylsulfatase were lower in the fumigated plots relative to the control soil. In the same field experiment, it was shown that repeated (over two consecutive years) soil fumigation with MeBr + CP significantly decreased the activities of acid phosphatase, β -glucosidase and dehydrogenase (Klose and Ajwa, 2004). Although enzyme activities in soils fumigated with PrBr, InLine, Midas and CP-EC were lower than in the control soil, differences were not significant (Klose and Ajwa, 2004), indicating that the studied MeBr alternatives had no long-term impact on microbial functionality.

In this work, the responses of enzyme-mediated processes and FAME profiles to fumigation suggest that there are differences in the effects of such treatments on the various components of the microbial community, and consequently, on the various functions of the soil biota in ecosystems. Enzyme activities can be used as an index of microbial functionality (Nannipieri et al., 2002), although extracellular enzymes may contribute considerably to the overall enzyme activity of a soil.

In this study, the extracellular fraction of acid phosphatase, arylsulfatase and β -glucosidase may have been stabilized by humic substances or clay minerals, and thus, partially protected from denaturing by fumigation. As documented elsewhere, this hypothesis, first reported by Ladd and Butler (1975), is supported by the high sensitivity of purified reference enzymes for acid phosphatase, β -glucosidase and arylsulfatase towards fumigation with MeBr+CP, PrBr, InLine, Midas and CP-EC (Klose and Ajwa, 2004) and chloroform (Klose and Tabatabai, 1999, 2002a,b).

In conclusion, changes in community composition and enzyme-mediated processes following fumigation are believed to affect the productivity of agricultural soils because hydrolytic enzymes regulate the rate at which organic materials are degraded and nutrients become available to plants. Since, the results of this study were obtained under laboratory conditions, some caution must be used in evaluating the relative effects of MeBr and alternative fumigants on soil microbial community structure and functionality. However, our findings provide a more complex insight into the biocidal activity of pesticide fumigants and their potential environmental impact that should be considered in the selection of potential MeBr replacements.

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